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(54) PROCESS OF PRODUCING PLATELET AGGREGATION INHIBITING SUBSTANCE FROM ALPINIA SPECIOSA

(57) ABSTRACT:

CONSTITUTION:

A process for producing a substance that inhibits platelet coagulation having steps comprising: extraction of a leaf body of Alpinia speciosa in ethyl acetate ester; fractionating the extract by the concentration gradient column chromatography utilizing silica gel as carrier and n-hexane ethyl acetate ester as solvent. The aggregation inhibiting substance includes quercetin and its glycoside.

ADVANTAGEOUS EFFECTS

The present invention provides a method of collecting platelet aggregation inhibitor with excellent yield, which is industrially advantageous.

Translator's note:

The specification uses the term "youtai" which may mean "leaf body" or "reticulate". "Reticulate" should correctly be spelled "mou-youtai" (veins of a leaf body) in Japanese, therefore, the translator translated "youtai" as "leaf body."

WHAT IS CLAIMED IS:

1. A process of collecting a platelet coagulation inhibitor having the steps comprising: extracting a leaf body of Alpinia speciosa with ethyl acetate ester; fractionating said elute by concentration-gradient column chromatography with solvent systems [in the order] of n-hexane ethyl acetate ester (10:1→1:1) → ethyl acetate ester → 80% aqueous methanol utilizing silica gel as a carrier; selecting a fraction having high platelet aggregation inhibition activity based on platelet aggregation inhibition activity levels as an index; and collecting the platelet aggregation inhibitor.

2. The process as set forth in Claim 1 wherein said Alpinia speciosa leaf body is an n-hexane extraction residue of a dried leaf body.

3. The process of collecting platelet aggregation inhibitor as set forth in Claim 1 wherein said collection of platelet aggregation inhibitor is the collection of quercetin; said quercetin is collected by means of separation of said fraction having a high platelet aggregation inhibition activity by concentration gradient silica gel column chromatography using solvents of n-hexane → ethyl acetate ester.

4. The process of collecting a platelet aggregation inhibitor as set forth in Claim 1 wherein said process has the steps comprising: fractionating said fraction having a high inhibition activity by concentration-gradient silica gel column chromatography using solvents [in the order of] n-hexane ethyl acetate ester (3:1 → 1:1) → ethyl acetate ester → methanol; selecting fractions having a high platelet aggregation inhibition activity from the resulting fractions; and collecting quercitrin (quercetin glycoside), in which rhamnose bonds to the 3rd position of quercetin, by means of separating said selected fractions by concentration gradiation silica gel column chromatography using solvents [in the order] of n-hexane-ethyl acetate ester (3:1 → 1:10) →ethyl acetate ester → methanol → 80% aqueous methanol.

DETAILED DESCRIPTION OF THE INVENTION

[0001]

TECHNICAL FIELD

This invention relates to a process of collecting a platelet aggregation inhibitor and its glycoside from Alpinia of Zingiberaceae (Gingiberaceae) with an excellent yield of the industrially advantageous level. The resulting quercetin and its glycoside provide a platelet aggregation inhibition activity and reduced vessel permeability, which are useful as drugs.

[0002]

RELATED ART

Alpinia speciosa (gettou) belongs to Alpinia of Zingiberaceae, which is an evergreen perennial herb that grows wild in the area covering the Okinawa Island to the southernmost end of Kyushu Island [in Japan]. Conventionally, Alpinia speciosa was used as an agent for controlling bugs (e.g. ticks) or fungus (e.g. mold) by extracting refined oil from the leaf body thereof with its distinct aroma. The stem thereof has been used as fibers as well. The inventors carefully studied the advanced areas in which Alpinia speciosa could be utilized and found that the extract from the leaf body thereof extracted with ethyl acetate ester strongly inhibits fibrinolytic activities and inhibits platelet aggregation. The inventors further studied ways to fractionate this extract using platelet aggregation inhibition activity as an index and found that quercetin and its glycoside provide very strong platelet aggregation inhibition activity. The inventors also found that Alpinia speciosa contains a large amount of quercetin and its glycoside, which can be produced by an industrially advantageous extraction method. The present invention is thus completed.

[0003]

OBJECT OF THE INVENTION

That is, the object of the invention is to provide a method of manufacturing from the leaf body of Alpinia speciosa, a platelet aggregation inhibitor, more specifically, quercetin and its glycoside, in an industrially advantageous manner.

[0004]

MEANS TO ACHIEVE THE OBJECTIVE

This invention provides a process of collecting a platelet coagulation inhibitor having the steps comprising: extraction of a leaf body of Alpinia speciosa with ethyl acetate ester; fractionating the extract by concentration gradient column chromatography with solvent systems

[in the order] of n-hexane-ethyl acetate ester (10:1→1:1) → ethyl acetate ester → methanol → 80% aqueous methanol utilizing silica gel as a carrier; selecting a fraction having high platelet aggregation inhibition activity; and collecting the platelet aggregation inhibitor. The present invention further provides a method of further fractionating the fraction to obtain quercetin and its glycoside as platelet aggregation inhibitor.

[0005]

As described above, Alpinia speciosa is a plant that belongs to Alpinia of Zingiberaceae, which includes Alpinia speciosa [typo: original text reads "pecionsa"] K. Schum., Alpinia urarensis Hay, Alpinia sanderae Sand, Alpinia oxyphylla L., Alpinia sp. and the like. Among all, Alpinia urarensis Hay contains a large amount of quercetin, which makes this spieces a desirable starting material. Quercetin is contained in leaf bodies thereof, therefore, quercetin was prepared from leaf bodies in the present invention. To enhance efficiency of extraction, sun drying or hot air drying of leaf bodies is desirable. Performing pre-extraction of Alpinia speciosa leaf body with n-hexane before ethyl acetate ester extraction is also desirable because this treatment removes impurities such as quercetin - like compounds and some other types. Accordingly, this pre-extraction increases the yield of quercetin.

[0006]

Next, the resulting n-hexane extract residue is extracted with ethyl acetate ester. 12kg-14kg of ethyl acetate ester by weight part per 2kg of dry leaf body is added to the extract and stirred at room temperature for several hours. Desirably, the n-hexane extract residue should be extracted several times. Quercetin and its glycoside are extracted in the ethyl acetate ester most completely in this way and the extract inhibits strong fibrinolytic activities. The extract may be used as it is or condensed and fractionated by the silica gel concentration gradient column chromatography with solvents [in the order] of n-hexane - ethyl acetate ester (10:1→ 1:1) → ethyl acetate ester → methanol → 80% aqueous methanol using the platelet aggregation inhibition activity (by the PRP-PPP method) as an index to collect a fraction having a strong platelet aggregation inhibition activity. This fraction is, then, used as a platelet aggregation inhibitor as it is or refined and dried to serve the purpose.

[0007]

Alternately, the above Fraction (I) is fractionated by concentration gradient chromatography in solvents of n-hexane → ethyl-acetate ester, using silica gel column so as to obtain a fraction having a high quercetin content, followed by collection of quercetin in the form

of crystals. Any methods normally used for crystallization of chemicals may be used for this crystallization; however, a method of recrystallization is more desirable. Moreover, Fraction (I) may be further fractionated by concentration gradient chromatography with a silica gel column utilizing solvents [in the order of] n-hexane - ethyl acetate ester (3:1 → 1:1) → ethyl acetate ester → methanol; selecting a fraction having high platelet aggregation inhibition activity from the resulting fractions; and collecting quercitrin in the crystalline form wherein rhamnose bonds to the 3rd position of quercetin by means of separating the selected fraction by concentration-gradiation silica gel column chromatography using solvents [in the order of] n-hexane- ethyl acetate ester (3:1 → 1:10) →ethyl acetate ester → methanol → 80% aqueous methanol. Any methods normally used for crystallization of chemicals can be used for this crystallization. Yet, methanol is used herein.

[0008]

The melting point, MS ^{1}H-NMR, and ^{13}C-NMR spectra of the crystals confirmed that the compounds obtained by the method of the present invention is quercetin and its glycoside as demonstrated in Examples below. Measurement methods illustrated in Examples below also confirmed that the resulting substances are platelet aggregation inhibitors having such platelet aggregation inhibition activity. These substances are thus useful as drugs that have platelet aggregation inhibition activity. The method of the present invention can yield quercetin at 0.2 - 0.3% of (dried) leaf body, and a quercetin glycoside at 0.3 to 0.4%.

[0009]

In the present invention, quercetin and its glycoside can be separated from other components at a high yield only by means of concentration gradient chromatography in which silica gel is used as a carrier utilizing the above solvents. Other types of means that use ion-exchange resins or other solvents in columns do not provide quercetin and its glycoside at a high yield.

[0010]

A platelet aggregation inhibitor obtained by this invention, which is quercetin or its glycoside, can be pharmaceutically prepared by a variety of methods for use in prevention or treatment of cerebral thrombosis, arteriosclerosis, myocardial infarction, and the like. Dosages may be changed in accordance with symptom, sex, age, and the like; however, a desirable dosage for an adult patient is 1 mg - 2g per day wherein the daily dosage should be divided into 1 - several portions for each administration. The substance may be pharmaceutically prepared for

oral administration in the form of powder, granule, tablet, capsule, syrup, or troche, or for parenteral administration in the form of intravenous injection, a subcutaneous injection, intramuscular injection, and the like. It may also be prepared for parenteral administration in the form of ointment, patches, suppository, and the like. Various additives (e.g. excipient, disintegrator, lubricant) may also be added to the preparation on an as needed basis. Furthermore, the substance obtained by this invention may be added to foods to give function such as prevention or treatment of cerebral thrombosis. Such function enhanced foods include noodles, breads, fish cakes, sausages, soups, ice creams, yogurts, juices or other drinks and the like.

[0011]

Next, examples of the present invention are described more concretely with reference to test examples.

EXAMPLE 1

COLLECTION OF QUERCETIN

Leaf bodies of Alpinia urarensis Hay were collected and dried under the sunlight. 36 liters of n-hexane were added to 5.1kg of the leaf bodies, and the mixture was refluxed at 70 °C 3 hours for extraction. The extraction was performed 3 times. 36 liters of ethyl acetate ester was further added to the residue and the mixture was refluxed at 60 °C for 3 hours for extraction. The extraction was performed 3 times and the extracts were collected. The extracts were vacuum dried to be concentrated at 50 °C, yielding 501g of concentrated dry substance. The extract of 501g was fractionated by the silica gel column chromatography. In other words, using the ∅ 8 x 90cm column containing a silica gel carrier, the extract was fractionated to 1 – 100 fractions by concentration gradient chromatograph using solvents arranged in the order of n-hexane - ethyl acetate ester (10:1→1:1) → ethyl acetate ester → methanol → 80% aqueous methanol. Then, Fraction 1 and Fraction 2, the earliest fractions, were fractionated again by concentration gradient silica gel chromatograph using n-hexane - ethyl acetate ester solvent. The effluent was collected and crystallized to give 1g of crystals.

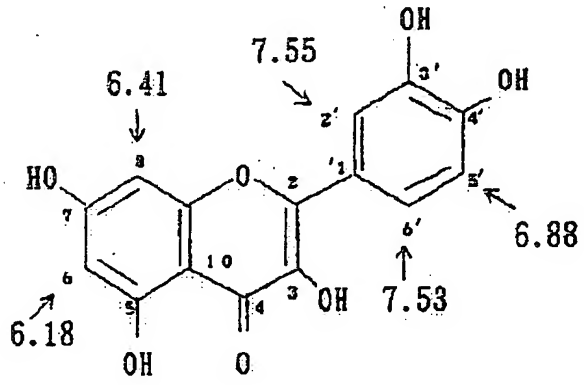
[0012]

The resulting crystals had a melting point of 123-125 °C (decomposition) and MS (m/z) of 302. The ^{13}C-NMR spectrum (DMSO-d6) was as follows:

C-2	146.8	C-1'	122.1
3	135.8	2'	115.2
4	175.8	3'	145.1
5	160.6	4'	147.7
6	98.4	5'	115.7
7	164.0	6'	120.1
8	93.5		
9	156.2		
10	103.1		

The physio-chemical property of the above substance was identical to that of quercetin. It was confirmed that the substance was quercetin having the following chemical structure.

FORMULA 1



wherein numerical values shown in the above formula are the chemical shift (DMSO-d6) values of the ^{1}HNMR spectrum of quercetin. Yield: 0.01% of dried leaf body.

[0013]

EXAMPLE 2

COLLECTION OF QUERCETIN GLYCOSIDE

Fraction 9 obtained by the method of Example 1 of a quercetin glycoside was applied to silica gel column to perform concentration gradient chromatography using solvents arranged in the order of n-hexane - ethyl acetate ester (3:1→ 1:1) → ethyl acetate ester → methanol to give Fractions 1-30. Fractions 17-21 were then applied to silica gel column to elute using solvents arranged in the order of n-hexane - ethyl acetate ester (3:1 →1:10) →. methanol → 80% aqueous methanol. The eluate was allowed to stand. The resulting crystals were collected by decantation

and recrystallized in methanol to give Crystal 2.

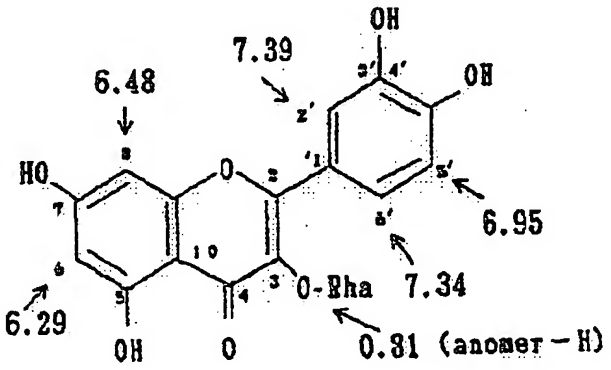
[0014]

Crystal 2 had a melting point of 177-180 ° C (dec) and MS (m/z) of 488. Its ^{13}C-NMR spectrum (DMSO-d6) values were as follows:

C-2	156.7	C-1"	102.2
3	134.6	C-2"	70.4
4	178.0	3"	70.8
5	161.6	4"	71.7
6	99.0	5"	70.6
7	164.4	6"	17.8
8	93.9	O-CH_3	
9	157.5		
10	104.5		
1'	121.4		
2'	115.9		
3'	145.4		
4'	148.7		
5'	116.1		
6'	121.1		

The physio-chemical property of the above substance was identical to that of a glycoside having rhamnose binding to the 3rd position of quercetin. It was confirmed that the substance was quercetin glycoside having the following chemical structure.

FORMULA 2



wherein numerical values shown in the above formula is the chemical shift (DMSO-d6) values of the ^{1}HNMR spectrum of quercetin glycoside.

[0015]

EXAMPLE 3

EFFECT OF QUERCETIN AND ITS RHAMNOSE GLYCOSIDE ON RABIT PLATELET AGGREGATION

The effect of quercetin obtained in Example 1 and rhamnose glycoside obtained in Example 2 on platelet aggregation were analyzed herein. In other words, rabbit platelet rich plasma-platelet poor plasma was introduced to analyze platelet aggregation inhibition ability by aggregometer (PAM-8T) using ADP (final concentration 2.0 μM) as an agonist in accordance with the PRP-PPP test method. Table 1 and Figure 1 illustrate the platelet aggregation inhibition abilities, indicating that quercetin and the rhamnose glycoside are useful as a platelet aggregation inhibitor, antithrombotic agent, anti-arteriosclerosis agent, cerebral thrombosis prevention or treatment agent, myocardial infarction prevention or treatment agent.

[0016]

TABLE 1

Compound	Compound concentration	Platelet aggregation ratio (%)
Control	0.5 mg/ml	0
Quercetin	0.5 mg/ml	-24
Glycoside	0.5 mg/ml	-90

The platelet aggregation inhibition ratio was computed by the following equation:

Platelet aggregation inhibition ratio (%) =

[(max. aggregation ability of sample / max. aggregation ability of control) -1] x 100.

These activity values are higher than that of aspirin, currently being prescribed as a platelet aggregation inhibitor, or a medicinal ginseng saponin.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the platelet aggregation inhibition abilities demonstrated in Example 3.

FIGURE 1